# Methods for Isolation of Triptolide Compounds from *Tripterygium wilfordii*

## Field of the Invention

The invention pertains to methods for extraction, isolation, and purification of triptolide and related molecules, such as tripdiolide and 16-hydroxytriptolide, from *Tripterygium wilfordii*.

## References

Z. Cheng *et al.*, "Research on extraction technology of *Tripterygium*", *Chinese J. of Pharmaceuticals* **21**(10):435-436 (1990).

S.M. Kupchan *et al.*, "Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*", *J. Am. Chem. Soc.* **94**(20):7194-7195 (1972).

S.M. Kupchan *et al.*, U.S. Patent No. 4,005,108 (1977).

P.E. Lipsky *et al.*, U.S. Patent No. 5,580,562 (Dec 1996).

K. Ren *et al.*, U.S. Appn. Pubn. No. 20040018260 (Jan 2004).

T.T. Wiedmann *et al.*, U.S. Patent No. 5,843,452 (Dec 1998).

C.P. Zhang *et al.*, "Studies on diterpenoids from leaves of *Tripterygium wilfordii*", *Acta Pharmaceutica Sinica* **28**(2):110-115 (1993).

## Background

Compounds derived from the Chinese medicinal plant *Tripterygium wilfordii* (TW) have been identified as having useful therapeutic properties, particularly immunosuppressive activity and anticancer activity. These compounds include triptolide, tripdiolide and 16-hydroxy triptolide. Synthetic derivatives and prodrugs of these compounds have also shown therapeutic activity, often in combination with improved pharmacological properties. See, for example, U.S. Patent Nos. 5,468,772 (Tripterinin compound and method), 5,648,376 (Immunosuppressant diterpene compound), 5,663,335 (Immunosuppressive compounds and methods), 5,759,550 (Method for suppressing xenograft rejection), 5,843,452 (Immunotherapy composition and method), 5,962,516 (Immunosuppressive compounds and methods), 6,150,539 (Triptolide prodrugs having high aqueous solubility), 6,294,546 (Uses of diterpenoid triepoxides as an antiproliferative agent), 6,537,984 (Uses of diterpenoid triepoxides as an antiproliferative agent), 6,548,537 (Triptolide prodrugs having high aqueous

solubility), 6,569,893 (Amino acid derivatives of triptolide compounds as immune modulators and anticancer agents), 6,599,499 (Uses of diterpenoid triepoxides as an antiproliferative agent), and 6,620,843 (Anticancer treatment using triptolide prodrugs), each of which is incorporated herein by reference.

Isolation of the native compounds from the plant material has, to date, typically required laborious extraction and purification procedures. Kupchan *et al.* (1972, 1977) describe a method in which the root material is extracted with ethanol, the solid extract is dissolved in ethyl acetate and partitioned with water, and the ethyl acetate fraction is chromatographed on silica gel. Cheng *et al.* (1993) describe a method in which the first extraction employs hot water, followed by addition of ethanol, filtration, removal of the ethanol, partitioning with chloroform, and chromatography on silica gel. The method described by Lipsky *et al.* (1996) employs subsequent extractions with chloroform, methanol, and toluene, with removal of solvent between each extraction, followed by chromatography on alumina and then on silica gel. Wiedmann *et al.* (1998) describe a method in which the root material is extracted with refluxing aqueous ethanol, the solid extract is partitioned between dichloromethane and water, and the dichloromethane phase is concentrated and chromatographed on silica gel. Ken *et al.* (2004) describe a method in which the root is extracted repeatedly with ethanol, and the extracts are concentrated and extracted repeatedly with chloroform, followed by chromatographic purification.

In isolation methods to date, the extraction steps generally produce an extract which retains large quantities of undesired materials, which then must be removed chromatographically, requiring large investments of time and materials. In view of the therapeutic utility of these compounds, higher efficiency methods for isolation and purification are desired.

## Summary of the Invention

The invention provides an improved method of isolating triptolide and related compounds, *e.g.* tripdiolide and 16-hydroxytriptolide, from *Tripterygium wilfordii* (TW) plant material. In accordance with the method, an extract of *Tripterygium wilfordii* plant material containing these compounds is formed and then purified. The extract is initially formed by

(a) extracting TW plant material, preferably root material, with aqueous ethanol, and concentrating to obtain a residue; and

(b) forming a slurry of this residue in an organic solvent, preferably a chlorinated hydrocarbon solvent, such as chloroform, methylene chloride, dichloroethane, or mixtures thereof; partitioning the slurry with water for a period of about 10 mins-10 hours; and then removing the water.

Typically, the extracting of step (a) includes three extractions with refluxing ethanol, each preferably using 4-5 mL of ethanol per g of plant material, followed by pooling of the extracts; the slurry formed in step (b) comprises 8-12 volumes of organic solvent relative to the residue; and the partitioning of step (b) employs 1/2 to 2 volumes of water relative to the slurry.

The subsequent purification comprises the steps of:

further partitioning the slurry with an aqueous solution of base, removing the aqueous solution of base, and removing at least a portion of the organic solvent from the slurry;

washing the residue with a lipophilic solvent; and

eluting the residue from a silica gel adsorbent.

In one embodiment, this purification comprises, following steps (a) and (b) above:

(c) partitioning the slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a further residue;

(d) washing the further residue with a hydrocarbon solvent, to obtain a solid product; and

(e) purifying the solid product by silica gel chromatography.

In another embodiment, this purification comprises, following steps (a) and (b) above:

(c) partitioning the slurry of the residue with an aqueous solution of base, removing the aqueous solution, and removing a portion of the organic solvent, to obtain a concentrated slurry;

(d) adding silica gel to the concentrated slurry, in an amount effective to adsorb the triptolide and related compounds;

(e) washing the residue and silica gel with a hydrocarbon solvent; and

(f) eluting the triptolide and related compounds from the silica gel.

In a further embodiment, this purification comprises, following steps (a) and (b) above:

(c) removing the organic solvent from the slurry of the residue;

(d) washing the residue with a hydrocarbon solvent;

(e) forming a further slurry of the washed residue in an organic solvent selected from chloroform, methylene chloride, dichloroethane and mixtures thereof;

(f) partitioning the further slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a solid product; and

(g) purifying the solid product by silica gel chromatography.

In the aqueous solution of base, the base is preferably a water soluble hydroxide, carbonate or bicarbonate having a counterion selected from lithium, sodium, potassium, cesium, ammonium, and tetraalkylammonium, where alkyl is preferably $C_1$-$C_4$ alkyl. Suitable bases include, for example, NaOH, KOH, $NaHCO_3$, $KHCO_3$, $Na_2CO_3$ and $K_2CO_3$. The solution may be selected, accordingly, from 0.1N - 2.5N aqueous NaOH, 0.1N - 2.5N aqueous KOH, 10% - 15% aqueous $NaHCO_3$, and 12% - 18% aqueous $KHCO_3$. The base partitioning is generally carried out for about 2 days, following a brief (*e.g.* 5-20 minutes, typically about 10 minutes) period of stirring. Optionally, following the removal of the aqueous solution of base, and prior to the removal of all or a portion of the organic solvent, the organic solvent is washed with a dilute aqueous acidic solution.

The lipophilic solvent is preferably a hydrocarbon solvent selected from linear, branched and cyclic hydrocarbons having 5-7 carbon atoms, and mixtures thereof; examples include hexane and cyclohexane. In one embodiment, the hydrocarbon solvent is hexane. The silica gel chromatography preferably employs a mobile phase comprising a nonpolar solvent, such as hexane, in combination with a more polar solvent, such as ethyl acetate.

## Detailed Description of the Invention

I. Overview

The invention provides a procedure for purifying an extract of *Tripterygium wilfordii* (TW) plant material containing triptolide and related compounds, such as

tripdiolide and/or 16-hydroxytriptolide, and isolating these compounds. Other useful related compounds such as triptriolide, tripchlorolide, and triptonide may also be isolated.

As described further below, the method includes treatment of an initial organic extract with aqueous base, which removes a significant amount of impurities at an early stage of the process, thereby increasing yield and reducing production costs. The base treatment effectively removes acidic or weakly acidic compounds (*e.g.* celastrol, triptoquinone A, triptoquinone G, 3-hydroxyoleanolic acid, polpunonic acid, tripterygic acid A, and phenolic compounds such as triptonoterpene, hypolide, triptophenolide, and triptonodial) from the organic TW extract. The base treatment also remove "oily" impurities from the extract via saponification and/or hydrolysis. This step has been found to remove about 70% of the undesired impurities from the extract, including oily materials whose removal typically generates a large majority of the cost of subsequent purification using prior art methods.

The method of the invention also includes an extraction with a lipophilic solvent, such as cyclohexane or a similar hydrocarbon-based solvent, *e.g.* hexanes, pentanes, petroleum ether, etc., to remove less polar impurities from the extract. This step further simplifies the subsequent chromatographic purification steps, by removing components which would otherwise typically be removed chromatographically.

II. Extraction Procedure

The *Tripterygium wilfordii* (TW) extract is initially formed by (i) extracting ground, chopped or otherwise finely divided TW plant material with aqueous ethanol, and concentrating the liquid extract to obtain a residue; (ii) forming a slurry of this residue in an organic solvent, preferably a chlorinated hydrocarbon solvent, typically selected from chloroform, methylene chloride, dichloroethane and mixtures thereof; (iii) partitioning the slurry with water for a period of about 10 mins-10 hours; and (iv) removing the water from the slurry.

The plant material may include the roots, stems, and leaves of *Tripterygium wilfordii*; preferably, the root material is used. The TW plant is found in the Fujiang Province and other southern provinces of China; TW plant material can generally be obtained in China or through commercial sources in the United States.

Preferably, the ethanol extraction (i) includes three extractions with refluxing ethanol, each using 4-5 mL of ethanol per gram of plant material, followed by pooling of the extracts. The amount of organic solvent used, typically chloroform or dichloroethane, used in step (ii) is generally about 8-12 times the volume of the residue from step (i). The partitioning step (iii) generally employs 1/2 to 2 volumes of water relative to the volume of slurry.

As used herein, "partitioning" of a mixture of two immiscible fluids generally refers to a short period of stirring, *e.g.* about 10-30 minutes, more typically 10-15 minutes, followed by settling of the mixture, typically for a period of hours or days. In this case, the organic slurry and water are first stirred together, *i.e.* for about 10 minutes, and allowed to settle over a period of about 10 mins - 10 hours, preferably about 2 hours.

III. Purification Procedure

In accordance with the method of the invention, the slurry obtained following step (iv) above is partitioned with an aqueous solution of base. In this process, the slurry and solution are first stirred together, *i.e.* for about 10 minutes, and allowed to settle over a period of about 10 mins-10 days, preferably about 1-4 days, more preferably about 2 days. The base is preferably a water soluble hydroxide, carbonate, or bicarbonate having a counterion selected from lithium, sodium, potassium, cesium, and ammonium. Suitable bases include, for example, NaOH, KOH, $NaHCO_3$, $KHCO_3$, $Na_2CO_3$ and $K_2CO_3$. In preferred embodiments, the aqueous solution of base is selected from 0.1-2.5N aqueous NaOH, 0.1-2.5N aqueous KOH, 10%-15% aqueous $NaHCO_3$, and 12%-18% aqueous $KHCO_3$.

The aqueous solution of base is removed, and then, optionally, the organic solvent is washed with a dilute aqueous acidic solution, *e.g.* 1% HCl. At least a portion of the organic solvent is then removed from the slurry.

The residue obtained, which may be substantially solid or a concentrated slurry, is then washed with a lipophilic solvent, followed by elution from a silica gel adsorbent. Preferably, the lipophilic solvent is a hydrocarbon solvent, preferably a saturated hydrocarbon, selected from linear, branched and cyclic hydrocarbons having 5-7 carbon atoms, and mixtures thereof. In one embodiment, the solvent is cyclohexane.

Note that in variations of the procedure, as described below, the order of certain treatment steps in the purification process may be altered.

Elution from a silica gel adsorbent (*i.e.* silica gel chromatography) preferably employs a solvent mixture, or mobile phase, comprising a non-polar solvent, such as a hydrocarbon, alkyl ether, or mixture thereof, in combination with a more polar solvent, such as an ester or ketone solvent. Such non-polar solvents include, for example, hexane, cyclohexane, petroleum ether, or THF. Such polar solvents include, for example, ethyl acetate, acetone, or methyl ethyl ketone (MEK). In one embodiment, the solvent mixture comprises cyclohexane and ethyl acetate. Solvent gradients may be used, in accordance with known methods.

IV. Variations on the Purification Procedure

In one embodiment, substantially all of the organic solvent is removed from the slurry following removal of the aqueous base, to give a solid or substantially solid residue. This residue is then washed with the lipophilic solvent to obtain a solid product, which is then purified by silica gel chromatography, as described above.

In another embodiment, only a portion of the organic solvent is removed from the slurry following removal of the aqueous base, to give a concentrated slurry. Silica gel is then added to the concentrated slurry, in an amount effective to adsorb triptolide and related compounds (*e.g.* tripdiolide and/or 16-hydroxytriptolide). The resulting mixture is then washed with the lipophilic solvent, and the triptolide and related compounds are then eluted from the silica gel.

In a further embodiment, extraction with the lipophilic solvent precedes the base treatment. Accordingly, prior to partitioning with base, the organic solvent is removed from the slurry obtained following step (iv) above, and the residue is washed with the lipophilic solvent. A further slurry of the washed residue is then formed, again in an organic solvent selected from chloroform, methylene chloride, dichloroethane and mixtures thereof, and this slurry is then partitioned with an aqueous solution of base, as described above, for a period of about 10 minutes to 10 days, preferably about 2 days. The aqueous base solution is removed, and substantially all of the organic solvent is then removed, to obtain a substantially solid residue, which is then purified by silica gel chromatography.

V. Exemplary Procedure

Following is an exemplary isolation procedure in accordance with one embodiment of the invention. This procedure is intended to illustrate and not to limit the invention.

A. Extraction

1. Dried TW biomass is ground into pieces (1 x 0.1 cm - 5 x 0.5 cm (length x diameter) for root core and stem; 0.1 – 2.0 cm in size (chip shape) for root bark. The ground TW biomass is refluxed with 50-95% (preferably 90%) ethanol for 2-5 (preferably 3) hours, 2-5 (preferably 3) times, at a weight/volume ratio of solid/ethanol of 1:4-6 (preferably 1:5) for the first extraction and 1:3-5 (preferably 1: 4) for the subsequent extractions.
2. The extracts are pooled, and the is ethanol removed under reduced pressure to give a dark slurry.

B. Isolation (including base treatment and hydrocarbon extraction)

1. The slurry is suspended in 8-12, preferably 10, volumes of dichloroethane or chloroform.
2. Water is added, in an amount of 1/2-2 volumes, preferably 1/2 volume, to the suspension. The mixture is stirred for about 10 minutes and allowed to settle over a period of 1-10 hours, preferably 2 hours.
3. The water layer is removed, and ½-1 volume, preferably 1/2 volume, of 0.1-2.5N, preferably 0.5 N NaOH or KOH solution, or 10-15% $NaHCO_3$, is added to the organic phase. The mixture is stirred gently for about 10 minutes, then left for 1-10 days, preferably 4 days, to allow the layers to separate.
4. The aqueous phase is removed.
5. Water is added, in an amount of 1/4-1 volume, preferably 1/4 volume, relative to the organic phase. The mixture is stirred for about 10 minutes and left for 1-3 hours. Optionally, the mixture is washed twice at this stage with 1% HCl.
6. The aqueous phase is removed, and a drying agent, such as $Na_2SO_4$ or $MgSO_4$ (3g/100mL), is added to the organic phase. The mixture is stirred and then filtered to remove the drying agent.
7. The organic solvent is removed completely under reduced pressure.

8. Cyclohexane is added to the resulting solid, and the mixture is stirred, *e.g.* for about 10 minutes, to suspend the solid.
9. The solid is removed by filtration and dried under reduced pressure at 40-60°C to obtain an intermediate product as a yellow powder.

C. Further Purification (silica gel chromatography)

1. The powder is dissolved in 1:1 cyclohexane:ethyl acetate at a concentration of 0.5-1.0 g/mL, preferably 0.75 g/mL.
2. The dissolved material is loaded onto a pre-equilibrated silica gel column (200-300 mesh, 100 x 1-20cm), using about 100g of silica gel per 1-3g, preferably per 2g, of the powder intermediate.
3. The product is eluted using the same solvent mixture at a flow rate of 10-30 ml/hr, preferably 18 ml/hour. Triptolide-enriched fractions are collected, monitoring with TLC or HPLC.
4. Triptolide-enriched fractions are pooled and the solvent removed.

Typically, triptolide and/or related compounds are crystallized from the obtained product by temperature adjustment and/or solvent (*e.g.* acetone or ethyl ether) adjustment. Optionally, column chromatography and/or crystallization are repeated.